REMARKS/ARGUMENTS

Status of the Claims

Claims 5-9, 13-17, 19, 20, 26-43, and 45-49 are pending in the present application. Claim 44 has been canceled without prejudice to or disclaimer of the subject matter contained therein. Claims 5, 13, 19, 36, 37, and 42 have been amended to remove "glycine." Claim 45 has been amended solely to correct dependency. No new matter has been added by amendment. Reexamination and reconsideration of the claims are respectfully requested.

These claim amendments were not presented earlier as Applicants earnestly believed that the previously presented claims recited patentable subject matter. Pursuant to 37 C.F.R. §1.116 and the *Manual of Patent Examining Procedure* (MPEP), any amendment that will place the application in condition for allowance may be entered after final rejection (MPEP § 714.12). Applicants believe that this amendment places claims 5-9, 13-17, 19, 20, 26-43, and 45-49 in condition for allowance. Accordingly, the Examiner is respectfully requested to enter these claim amendments to further prosecution or to place the application in better condition for appeal.

The Examiner's comments in the Office Action are addressed below in the order set forth therein.

The Rejection Under 35 U.S.C. §103(a) Should be Withdrawn

The Examiner has maintained the rejection of claims 5-9, 13-17, 19, 20, and 26-49 under 35 U.S.C. §103(a) on the grounds that they are unpatentable over Dorin *et al.* (U.S. Patent No. 5,814,485; hereinafter the '485 patent) in view of Hershenson *et al.* (U.S. Patent No. 5,004,605; hereinafter the '605 patent) and further in view of *The Merck Index* (11th Edition, 1989, Merck and Co., p. 132). Claim 44 has been canceled, rendering this rejection moot as applied to this claim. This rejection is respectfully traversed as applied to the remaining claims.

Applicants have discovered improved pharmaceutical formulations including monomeric interferon-beta (IFN-β) having a pH of about 3.0 to about 5.0 in an aspartic acid or sodium succinate buffer. Applicants have previously made of record, and the Examiner has acknowledged (Office Action, dated November 8, 2004), that the '485 patent fails to teach either

the claimed pH range, or aspartic acid or sodium succinate. The Office Action contends that it would have been obvious to one of ordinary skill in the art at the time the invention was made to combine the teachings of the '485 patent, concerning monomeric IFN-β at a pH of 6.0 to 7.5, with the teachings of the '605 patent, concerning IFN-β at a pH of 2.0 to 4.0, and further with the teachings of *The Merck Index*, providing pKa data for aspartic acid and sodium succinate. Accordingly, there must be some suggestion in these cited references or knowledge generally available to one of ordinary skill in the art to modify these cited references to arrive at Applicants' claimed invention, wherein monomeric IFN-β is formulated with aspartic acid or sodium succinate in a pH range of about 3.0 to about 5.0.

The '485 patent teaches that "maintenance of pH is **critical** to prevent such physical and chemical alterations, such as oxidation, during storage of the IFN-β polypeptide. The pH will be chosen not only to optimize the longevity of the IFN-β polypeptide but to ease administration of the IFN-β polypeptide to humans" (column 13, lines 46-51, emphasis added). As discussed above, the '485 patent teaches that the pH of the IFN-β formulation is adjusted to between 6.0 and 7.5 (column 13, line 52). Applicants respectfully submit that there is no suggestion or motivation to combine the teachings of the '485 patent concerning monomeric IFN-β formulated at a pH of 6.0 to 7.5, with the teachings of the '605 patent, concerning IFN-β formulated at a pH of 2.0 to 4.0.

It is well settled in the case law that obviousness can only be established by combining or modifying the teachings of the prior art to produce the claimed invention where there is some teaching, suggestion, or motivation to do so found either explicitly or implicitly in the references themselves or in the knowledge generally available to one of ordinary skill in the art. "The test for an implicit showing is what the combined teachings, knowledge of one of ordinary skill in the art, and the nature of the problem to be solved as a whole would have suggested to those of ordinary skill in the art." *In re Kotzab,* 217 F.3d 1365, 1370, 55 USPQ2d 1313, 1317 (Fed. Cir. 2000). The mere fact that references can be combined or modified does not render the resultant combination obvious unless the prior art also suggests the desirability of the combination. *In re Mills*, 916 F.2d 680, 16 USPQ2d 1430 (Fed. Cir. 1990). Applicants respectfully submit that the '485 patent does not motivate the skilled artisan to combine its teachings with the teachings of

the '605 patent to arrive at the improved pharmaceutical formulations including monomeric IFN-β having a pH of about 3.0 to about 5.0 that Applicants have discovered. The combination of the teachings of the '485 patent (pH range of 6.5 to 7.5) with the '605 patent (teaching the use of glycine to formulate IFN-β) leads to an IFN-β formulation using glycine at a pH range of 6.0 to 7.5. Because the first pKa of glycine is 2.34 (see *The Merck Index*), glycine is not a suitable buffer for formulations with a pH range of 6.0 to 7.5. Applicants maintain that the '485 patent teaches away from such combination. Nevertheless, to expedite prosecution of this case, Applicants have amended the claims to delete glycine.

Furthermore, Applicants respectfully submit that even if such an impermissible combination were made, there is no suggestion or motivation to further combine the teachings of *The Merck Index* concerning pKa data for aspartic acid and sodium succinate. It is readily apparent that the reference to aspartic acid and sodium succinate, within the context of a description of their respective physico-chemical properties in *The Merck Index*, merely invites experimentation; yet an invitation to experiment is not sufficient grounds to reject an invitation as obvious. For example, in a list of critical variables to be considered in protein refolding, Fiona *et al.* (In *Guide to Protein Purification*, ed. by Deutscher, MP., Ch. 20, Academic Press, Inc., 1990, pp. 264-76; attached as **Exhibit A**) place pH at the top of the list (see page 270). Consequently, a general description of the physico-chemical properties of aspartic acid and sodium succinate (as found in *The Merck Index*) does not guide the skilled artisan to their recited use in improved pharmaceutical formulations including monomeric IFN-β having a pH of about 3.0 to about 5.0, as discovered by the Applicants. Where the prior art gives only general guidance as to the particular form of the invention or how to achieve it, as here, obviousness may not be found. *Hybritech, Inc. v. Monoclonal Antibodies, Inc.*, 802 F.2d 1367, 231 USPQ 81, 90-91 (Fed. Cir. 1986).

In view of the above arguments, Applicants contend that a *prima facie* case of obviousness under 35 U.S.C. §103(a) has not been established. Accordingly, reconsideration and withdrawal of the rejection are respectfully requested.

CONCLUSION

In view of the foregoing amendments and remarks, Applicants respectfully submit that the rejection of the claims under 35 U.S.C. §103(a) is overcome. Applicants respectfully submit that this application is now in condition for allowance. Early notice to this effect is solicited. If in the opinion of the Examiner, a telephone conference would expedite the prosecution of the subject Application, the Examiner is invited to call the undersigned attorney.

It is not believed that extensions of time or fees for net addition of claims are required, beyond those that may otherwise be provided for in documents accompanying this paper. However, in the event that additional extensions of time are necessary to allow consideration of this paper, such extensions are hereby petitioned under 37 CFR § 1.136(a), and any fee required therefore (including fees for net addition of claims) is hereby authorized to be charged to Deposit Account No. 16-0605.

Respectfully submitted,

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Nora C. Martinez

Methods in Enzymology

Volume 182

Guide to Protein Purification

EDITED BY

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4. Solubilization of Protein. Experimental points should be determined in triplicate. An aliquot of 0.5 ml of the stock membrane suspension is pipetted into each tube at 4°. An aliquot of 0.5 ml of the differing detergent concentrations at twice their final concentration to be tested is then pipetted into appropriate tubes. Tubes are maintained at 4° for 1 hr, and then centrifuged at 105,000 *g* for 1 hr at 4°. Tubes may be stirred during the incubation period, but excessive agitation should be avoided since foam formation is associated with the denaturation of proteins.

5. Determination of Solubilized Protein. After centrifugation, the clear supernatant should be removed from any residual pellet. Protein determinations should be made both on the solubilized protein and any pellet after suspension in an equal volume of solubilization buffer. Some pellets are difficult to resuspend, and may require the use of a small tissue homogenizer. Aliquots of the solubilized protein and the resuspended pellet can be assayed for the individual protein or activity being studied.

Conclusion

The discussion presented here provides a few relatively simple guidelines for the selection of detergents and their use in the solubilization of membrane proteins in a native state. This is truly a task about which few valid generalizations may be made. For this reason, general recipes concerning the total amount of detergent to be used, or specific suggestions of "nondenaturing" detergents which will be useful in all situations, cannot be made.

[20] Solubilization of Protein Aggregates

By FIONA A. O. MARSTON and DONNA L. HARTLEY

Introduction

Major advances in genetic engineering have resulted in the development of bacterial cell systems, especially *Escherichia coli,* capable of producing high levels of proteins expressed from cloned genes.[1,2] In *E. coli,* in a majority of cases, the overexpressed protein accumulates intracellularly in an insoluble form, resulting in phase-bright inclusions in

[1] J. F. Kane and D. L. Hartley, *Trends Biotechnol.* **6,** 95 (1988).
[2] F. A. O. Marston, *Biochem. J.* **240,** 1 (1986).

the cytoplasm.[3-5] Solubilization of these protein aggregates is the subject of this chapter.

From a purification standpoint, the accumulation of protein in an aggregated form is advantageous. After breaking open the cells and centrifuging the resulting lysate the aggregated protein can be recovered in the pellet fraction about 50% pure, although mostly in an inactive form. The protein in the inclusion bodies can be a mixture of monomeric and multimeric forms,[6] both reduced and oxidized.[2] The major problem then becomes one of recovering biologically active protein in high yield. In order to accomplish this the protein in the inclusion bodies must be solubilized, refolded, and purified, in a specific order.[2]

The common stages in processes designed to recover biologically active, soluble protein from such aggregates include (1) cell lysis, (2) isolation of inclusion bodies, (3) solubilization of protein in inclusion bodies, and (4) refolding of solubilized protein.

In the following sections we first present general approaches used to isolate and solubilize protein aggregates. Upon refolding such methods may yield active soluble protein; however, there are many factors influencing the final yield, each dependent on the nature of the protein itself. The later sections of the chapter illustrate these factors by reference to the processes optimized for specific proteins.

General Considerations

Protein inclusion bodies in *E. coli* are formed during high-level expression of cloned genes.[1,2] Exactly why inclusion bodies form is not known, but there are parallels between such cells and cells in which the heat-shock system has been induced.[7-9] The majority of protein contained within these inclusion bodies is in a denatured form, in part due to the reducing environment of the *E. coli* cytoplasm.[10] In addition, dimers and higher molecular weight multimers may be present. Hydrophobic interactions between regions of the unfolded protein molecules in the inclusion bodies may also be important. That the protein is not simply precipitated due to a high localized concentration is evidenced by the fact that strong

[3] D. C. Williams, R. M. Van Frank, W. L. Muth, and J. P. Burnett, *Science* **215**, 687 (1982).
[4] R. G. Schoner, L. F. Ellis, and B. E. Schoner, *Bio/Technology* **3**, 151 (1985).
[5] L. A. Holladay, R. G. Hammons, Jr., and D. Puett, *Biochemistry* **13**, 1653 (1974).
[6] D. F. Marks, S. D. Lu, A. A. Creasey, R. Yamamoto, and L. S. Lin, *Proc. Natl. Acad. Sci. U.S.A.* **81**, 5662 (1984).
[7] D. L. Hartley and J. F. Kane, *Biochem. Soc. Trans.* **16**, 101 (1988).
[8] W. F. Prouty, M. J. Karnovsky, and A. L. Goldberg, *J. Biol. Chem.* **250**, 1112 (1975).
[9] C. A. Schachtele, D. L. Anderson, and P. Rogers, *J. Mol. Biol.* **33**, 861 (1968).
[10] C. K. Tuggle and J. A. Fuchs, *J. Bacteriol.* **162**, 448 (1985).

TABLE I
REAGENTS THAT RELEASE EUKARYOTIC POLYPEPTIDES FROM INCLUSION BODIES INTO SOLUTION[a]

Reagent	Eukaryotic polypeptide solubilized
Guanidine-HCl (5–8 *M*)	Insulin A and B chains
	Bovine growth hormone (bGH)
	Urokinase
Urea (6–8 *M*)	Prochymosin
	Interferon
	Salmon growth hormone
SDS	Interferon
	Interleukin 2 (IL-2)
Alkaline pH (>9.0)	Prochymosin
	Chicken growth hormone
Acetonitrile/propanol	T4 *regA* protein

[a] Reproduced from Ref. 21.

"solvents" are necessary to solubilize the proteins,[2] as illustrated in Table I.

Once proteins are solubilized, the task becomes one of refolding the protein molecules into their correct conformations. This can be accomplished by removing the solubilizing agent and replacing it with an aqueous buffer (e.g., by dialysis or dilution). The situation is somewhat more complicated in proteins containing disulfide bridges. Oxidation of the reduced protein must occur either prior to or concomitant with refolding.

The concentration of protein in the refolding solution also affects the yield of recoverable active protein.[11] The most significant loss during refolding of concentrated protein solutions is due to aggregate formation, which is frequently due to covalent modifications of the unfolded protein molecules, such as intermolecular disulfide formation.[11] It is therefore recommended that refolding be carried out at as dilute a concentration as feasible, taking into consideration workable volumes of solutions and possible loss due to high dilution for subsequent purification steps.

Finally, purification is usually effected to remove other proteins and contaminating nucleic acids also present in the inclusion body,[7] as well as to remove incorrectly refolded forms of the protein of interest. Depending on the specific characteristics of the protein to be purified, conditions can often be defined so that an ion-exchange column can effectively remove

[11] A. Light, *Bio/Technology* **3**, 298 (1985).

nucleic acid contaminants and proteins of very different isoelectric points. Subsequent purification is frequently carried out by HPLC.[2,12] With very low level expression, i.e., very small inclusion bodies, more impurities will be present than when starting with large inclusion bodies,[13] and will undoubtedly have a negative impact on the efficiency of solubization and refolding. This emphasizes the importance of optimizing expression either by genetic manipulations or by amelioration of fermentation and induction parameters, before attempting to purify significant quantities of the expressed protein.

General Solubilization Scheme

Preparation of Certain Solubilization Reagents

Urea (6 M). Ultrapure-grade urea should be used, with deionized distilled water. This solution should be deionized by passage through a mixed bed ion-exchange resin and stored at 4°. Cold storage will reduce the formation of cyanate ions, which can ultimately react with amino groups to form carbamylated derivatives.

Guanidine-HCl (6 M). Ultrapure grade guanidine is used and the pH of the final solution is adjusted to 7–8 with concentrated HCl.

Cell Disruption

There are several ways to recover protein inclusion bodies from pelleted bacterial cells. The most frequently used are sonication[12,14,15] and passage through a French pressure cell,[16,17] but it is also possible to use the lysozyme–detergent treatment.[18]

Sonication. Suspend 15 g of cell paste in 50 ml of chilled deionized water. Sonicate two times for 45 sec each at 0°, using 50% power. Keep cell suspension on ice.

[12] Y. Saito, Y. Ishi, M. Niwa, and I. Ueda, *J. Biochem.* (*Tokyo*) **101**, 1281 (1987).
[13] D. N. Brems, S. M. Plaisted, H. A. Havel, E. W. Kauffman, J. D. Stodola, L. C. Eaton, and R. D. White, *Biochemistry* **24**, 7662 (1985).
[14] S. M. Zurawski, T. R. Mosmann, A. Benedik, and G. Zurawski, *J. Immunol.* **137**, 3354 (1987).
[15] J. Bartholomé-DeBelder, M. Nguyen-Disteche, N. Houba-Herin, J. M. Ghuysen, I. N. Maruyama, H. Hara, Y. Hirota, and M. Inouye, *Mol. Microbiol.* **2**, 519 (1988).
[16] D. V. Goeddel, D. G. Kleid, F. Bollivar, H. L. Heyneker, D. G. Yansura, R. Crea, T. Hirose, A. Kraszewski, A. Itakura, and A. D. Riggs, *Proc. Natl. Acad. Sci. U.S.A.* **76**, 106 (1979).
[17] G. Schumacher, D. Sizmann, H. Huag, and A. Bock, *Nucleic Acids Res.* **14**, 5713 (1986).
[18] J. A. Goliger and J. W. Roberts, *J. Biol. Chem.* **262**, 11721 (1987).

French Press. Prechill the French press cell to be used. Suspend 15 g of cell paste in 30 ml of chilled deionized water. Pass the cell suspension through the cell twice at 5000 lb/in. pressure. Keep the suspension on ice.

Centrifugation and Washing

Centrifuge the suspension resulting from cell breakage at 10,000 to 20,000 *g* for 10 to 20 min at 4°. (At this point it is prudent to look at a sample of the supernatant under the microscope to determine if a significant quantity of inclusion bodies remain. This can vary depending on the protein being expressed by the cells. If significant amounts of inclusion bodies remain in the supernatant, recentrifuge at 20,000–25,000 *g* for 10–15 min.) Carefully decant the supernatant and resuspend the pellet in 50 ml of chilled deionized water.

Washing Inclusion Bodies

The fact that many of the eukaryotic proteins expressed in *E. coli* are insoluble is an advantage since isolation of inclusion bodies in itself can be a very efficient purification step. However, to a varying degree, protein contaminants do remain after inclusion body isolation by centrifugation coupled with washing procedures. Such contaminants may subsequently interfere with refolding or may prevent it altogether. Most, if not all, of these contaminating proteins are strongly associated with or entrapped in the inclusion bodies and therefore may require solubilization under the type of conditions listed in Table I to release them.

It is frequently advantageous to wash the inclusion bodies with a solution other than water. In this case, the wash step can be carried out as above, usually with the incorporation of some type of detergent in the washing buffer.[15,19] It has also been found that treatment of the broken cell pellet with 0.2 mg ml^{-1} lysozyme/1 m*M* EDTA/1 mg ml^{-1} deoxycholate, while the protein is still insoluble, will remove the majority of nucleic acids, phospholipids, and lipopolysaccharides from bovine somatotropin inclusion bodies, without solubilizing the protein.[19] A similar wash, using lysozyme/EDTA/DNase/Nonidet was also found to be effective in selectively removing contaminants without solubilizing the protein in the inclusion bodies.[15]

Denaturants can in some cases be used to solubilize contaminants preferentially. Directly expressed bovine growth hormone (bGH) forms inclusion bodies in *E. coli*, and a number of contaminants copurify with

[19] K. E. Langley, T. G. Berg, T. W. Strickland, D. M. Fenton, T. C. Boone, and J. Wypych, *Eur. J. Biochem.* **163**, 313 (1987).

the aggregated bGH.[4] Washing the inclusion bodies with up to 4 *M* urea only solubilizes the contaminants, but as the urea concentration is increased to 5 *M* the bGH is partially solubilized as well.

Solubilization

Typical classes of reagents which can be used to solubilize proteins from inclusion bodies are listed in Table I. In general a solubilization and refolding protocol for protein aggregates in *E. coli* will be protein specific. At each stage there are variables which are critical and must be considered. For the solubilization stage these variables are as follow:

1. pH
2. Incubation temperature
3. Time of exposure to solvent
4. Ionic components of the solvent
5. Concentration of the solubilization agent
6. Concentration of total protein
7. Ratio of solubilization agent to protein
8. Presence or absence of redox agents
9. Derivatization of thiol groups

Having established the optimum reagent for solubilization, the other variables listed must be investigated in conjunction with refolding to determine the most efficient overall process. A recommended strategy is to screen the variables first on a small scale (1- to 2-ml working volume) in order to select a limited number of variables to evaluate on a larger scale.

Commonly denaturants are used early in the evaluation of solubilization agents. A typical method might be as follows:

Using a clean spatula, remove the washed inclusion body paste to a preweighed glass beaker and determine its weight. Add a stir bar and use a magnetic stir plate on the lowest speed possible. Slowly add a solution of 6 *M* urea or 6 *M* guanidine chloride to give a final protein concentration of 1–2 mg/ml. Continue stirring at the lowest speed until the paste is dissolved. It is frequently desirable to add a thiol reagent, such as cysteine or 2-mercaptoethanol, to the urea or guanidine-HCl solution to reduce all of the protein present in the inclusion bodies.

For example, it has been estimated that only 80% of the bGH in inclusions is in a reduced form.[19] In order to recover active protein from the remaining oxidized material, it is necessary to reduce it, thus starting with 100% reduced material (i.e., random coil). In contrast, solubilization

of prochymosin (in urea or guanidine-HCl) in the presence of thiol reagents has a negative impact on the recovery of active protein.[20]

If the solution resulting from solubilization is quite cloudy, it is recommended that a centrifugation step be added here. Centrifuge the protein solution at 20,000–30,000 *g* for 15 min. Decant the cleared supernatant to a fresh beaker.

Refolding

Protein aggregates in *E. coli* must be first solubilized and then refolded in order to regain active protein. These two processes, solubilization and refolding, are interdependent and their use in recovering proteins from aggregates in *E. coli* has been reviewed.[2,21] As for solubilization, there are variables at the refolding stage which are critical and must be considered:

1. pH
2. Incubation temperature
3. Time
4. Ionic components of the solvent
5. Rate of change from solubilization to refolding solvent conditions
6. Purity of the protein of interest
7. Concentration of the protein of interest
8. Presence or absence of redox agents

It is pertinent in this chapter to consider the key points relating to solubilization which impact on refolding.

It is important during both solubilization and refolding to minimize exposure to conditions which result in derivatization of amino acid side chains (e.g., pH values of greater than 9.0). During the refolding stage perhaps the key parameters influencing recovery are purity and concentration of the protein of interest. The concentration of the protein should be such that at the incubation temperature used, intramolecular bonds form in preference to intermolecular bonds.

Finally, for proteins which contain cysteine residues and in their native form contain disulfide bonds, the redox conditions during solubilization and refolding may be critical. Strict guidelines cannot be provided since requirements are protein specific. Prochymosin, for example, contains six cysteine residues which form three disulfide bonds in the native protein. If aggregated, prochymosin isolated from *E. coli* is first fully

[20] F. A. O. Marston, P. A. Lowe, M. T. Doel, J. M. Schoemaker, S. White, and S. Angal, *Bio/Technology* **2**, 800 (1984).

[21] F. A. O. Marston, *in* "DNA Cloning" (D. Glover, ed.), Vol. 3, p. 59. IRL Press, Oxford, 1987.

reduced, and then solubilized in a strong denaturant (8 *M* urea). However, little if any activity is recovered upon refolding. The best yields of active protein could be obtained by omitting redox reagents at both the solubilization and refolding stages.[20]

In contrast, there are examples of proteins which require redox reagents at the solubilization stage, or refolding stage, or both. One such example is interleukin 2 (IL-2) (three cysteine, one disulfide) which is in a fully reduced form in inclusion bodies.[22] At the solubilization stage it apparently is essential to maintain the denatured IL-2 in a reduced form.[23] In both this study and another in parallel,[22] it was then clearly demonstrated that oxidative refolding is required to obtain biologically active IL-2.

Solubilization of Hybrid Protein Aggregates

Recombinant DNA technology can be used to produce hybrid proteins by fusion of the coding sequence of the gene of interest to one or more other gene sequences. This is a common strategy used to improve expression levels. The mechanism by which expression levels are increased can differ and may be the result of reduced proteolysis[24] or sequestration of the gene product into inclusion bodies.[25]

Cleavage of the hybrid protein *in vitro* may be required in order to generate the protein of interest. The cleavage strategy must be considered in advance of gene construction and an appropriate cleavage site engineered immediately before the N-terminus (or after C-terminus) of the coding sequence of the protein of interest. Such a cleavage site must either be absent in the required protein or, if present, must be less reactive than the sequence located at the junction. Essentially, there are two methods of cleavage which can be employed: chemical or enzymatic. When the hybrid protein is aggregated and insoluble such cleavage must be performed under conditions which solubilize the protein and make the cleavage site accessible.

Extreme acid conditions can solubilize polypeptides and concomitantly catalyze hydrolysis. Conditions can be adjusted such that hydrolysis occurs at specific amino acid sequences, typically after Asp. For native proteins partial acid hydrolysis is typically performed in 30 m*M* HCl,

[22] T. Tsuji, R. Nakagawa, N. Sugimoto, and K.-I. Fukuhara, *Biochemistry* **26**, 3129 (1987).
[23] M. P. Weir and J. Sparks, *Biochem. J.* **245**, 85 (1987).
[24] K. Itakura, T. Hiroso, R. Crea, A. D. Riggs, H. L. Heyneker, F. Bolivar, and H. W. Boyer, *Science* **198**, 1056 (1977).
[25] S.-H. Shen, *Proc. Natl. Acad. Sci. U.S.A.* **81**, 4627 (1984).

TABLE II
CONDITIONS USED FOR CNBr CLEAVAGE OF CERTAIN AGGREGATED HYBRID PROTEINS

Hybrid protein	Percentage formic acid (v/v)	CNBr : protein (w/w)	Incubation time (hr)	Protein concentration (mg ml^{-1})	Ref.
Tandem-linked proinsulin or β-galactosidase–proinsulin	70	50	35	—	25
bcZ–substance P	88	10	12	15.0	29
β-galactosidase–growth hormone	44	5	3	20.0	30

in vacuo at 105° for 20 hr.[26] As an example, acid hydrolysis was used to release bGH from fusions to the *trpE* and *trpLE* gene products.[27]

However, acid conditions alone were not adequate to solubilize aggregated proteins for cleavage. The suspending medium required was 70% formic acid containing the denaturant guanidine hydrochloride at a concentration of 6 *M*.[27] The proteins were incubated at concentrations of between 0.85 and 1.0 mg ml^{-1} for 72 hr at 37° to allow cleavage to occur.

When the protein of interest lacks Met residues in its sequence, a common strategy is to construct the fusion protein with a Met residue at the junction between the two parts of the hybrid molecule. Then cyanogen bromide (CNBr), which selectively cleaves proteins immediately after Met residues, can be used to cleave the hybrid *in vitro*. CNBr cleavage is performed under acid conditions (commonly formic acid), and therefore aggregated proteins may be solubilized.[28]

Key parameters to consider when establishing hydrolysis conditions include protein concentration, formic acid concentration, ratio of CNBr to protein (w/w), temperature, and time. Table II[29,30] illustrates, with a few examples of insoluble hybrid proteins, the wide range of conditions which have been employed. In each of these examples, the acid conditions alone were adequate for the CNBr to gain access to the cleavage site.

When cleavage of hybrid proteins is to be catalyzed enzymatically, the

[26] R. L. Lundblad and C. M. Noyes, *in* "Chemical Reagents for Protein Modification," Vol. 1. CRC Press, Boca Raton, Florida, 1984.
[27] P. R. Szoka, A. B. Schreiber, H. Chan, and J. Murphy, *DNA* **5**, 11 (1986).
[28] E. Gross, this series, Vol. 11, p. 27.
[29] T. Kempe, S. B. H. Kent, F. Chow, S. M. Peterson, W. I. Sundquist, J. J. L'Italien, D. Harbrecht, D. Plunkett, and W. J. DeLorbe, *Gene* **39**, 239 (1985).
[30] T. Kempe, F. Chow, S. M. Peterson, P. Baker, W. Hays, G. Opperman, J. J. L'Italien, G. Long, and B. Paulson, *Bio/Technology* **4**, 565 (1986).

hybrid should be purified to >80% homogeneity (see following section) to maximize the efficiency of the proteolytic step. There are two possible proteolytic strategies for aggregated hybrids:

1. Solubilize the hybrid proteins and cleave in the presence of the solubilization agent.
2. Refold the solubilized hybrid, removing the solubilization agent, and then cleave.

Strategy (1) involves the use of balanced conditions which are severe enough to dissociate aggregates but which do not inactivate the proteolytic enzyme. There are certain proteases which are active in the presence of high levels of denaturant, e.g., clostripain (4 *M* urea[31]) and carboxypeptidase (5 *M* urea[32]). In an example of this strategy fusion between the bacterial enzyme chloramphenicol acetyltransferase (CAT) and human calcitonin (hCT) was engineered with a -Lys-Arg- cleavage site,[31] which can be cleaved by clostripain. The hybrid CAT-Lys-Arg-hCT was purified by isolation and washing of inclusion bodies. The washed inclusions were then solubilized in 100 m*M* Tris-HCl, pH 7.8, containing 8 *M* urea and 0.14 *M* 2-mercaptoethanol at a final protein concentration of 40 mg ml^{-1}. The suspension was incubated at 37° for 10 min and then diluted 1 : 1 (v/v) with water. Clostripain was then added to a final ratio of protease : fusion protein (w/w) of 1 : 40 and the suspension was incubated for a further 15 min at 37° before the addition of trifluoroacetic acid [5% (v/v), final concentration] to stop the enzymatic reaction.

With strategy (2), in which hybrid proteins are solubilized and refolded before cleavage, the potential problem is that the components of the hybrid may interact and therefore prevent correct folding. However, the strategy has been used successfully with fusions between the λ*cII* gene product and β-globin.[33] The fusion protein is isolated in washed inclusion bodies that were solubilized in 8 *M* urea and purified (see below). Then dialysis is used to remove the denaturant and allow refolding. Cleavage of the fusion protein is then catalyzed with blood coagulation factor Xa, approximately 160 mg of fusion protein being cleaved with 5 mg of activated factor Xa.

Purification of Solubilized Protein Aggregates

Once the typical washing procedures described earlier have been used effectively to purify inclusion bodies, the only method to dissociate the

[31] P. A. Lowe, S. K. Rhind, R. Sugrue, and F. A. O. Marston, *Protein Purif.: Micro Macro–UCLA Symp. Mol. Cell. Biol.* **68**, 429 (1987).
[32] H. M. Sassenfeld and S. J. Brewer, *Bio/Technology* **2**, 76 (1984).
[33] K. Nagai, M. F. Perutz, and C. Poyart, *Proc. Natl. Acad. Sci. U.S.A.* **82**, 7252 (1985).

TABLE III
CHEMICAL STABILITY OF SELECTED CHROMATOGRAPHY MATRICES

Chromatographic mode	Matrix	Stability			Supplier
		pH	Denaturants	Detergent	
Ion exchange	Mono-S	2–12	8 *M* urea	Nonionic, anionic, zwitterionic	Pharmacia
Ion exchange	Mono-Q	2–12	8 *M* urea	Nonionic, cationic, zwitterionic	Pharmacia
Ion exchange	TSK-DEAE 5PW	2–12	8 *M* urea, 6 *M* GuHCl	All	Toyo-Soda
Ion exchange	DEAE-Sephacel	2–12	8 *M* urea, 6 *M* GuHCl	Nonionic, anionic	Pharmacia
Gel filtration	Sepharose CL	3–14	8 *M* urea, 6 *M* GuHCl	All (sodium deoxycholate not recommended)	Pharmacia
Gel filtration	Superose	2–12	8 *M* urea, 6 *M* GuHCl	All (sodium deoxycholate not recommended)	Pharmacia
Hydrophobic interaction	Phenyl-Superose	2–12	8 *M* urea	Nonionic, cationic, zwitterionic	Pharmacia
Hydrophobic interaction	TSK Phenyl 5PW	2–12	8 *M* urea, 6 *M* GuHCl	All	Toyo-Soda
Reversed phase	PLRP-S	1–13	Urea, GuHCl	All	Polymer Laboratories
Reversed phase	Ultrapore short chain C3	2.5–7.5	Urea, GuHCl	All	Beckman

remaining contaminating proteins may be total solubilization using the harsh conditions listed in Table I. However, it is still possible to purify the protein of interest using conventional chromatography matrices, which are stable under such extreme conditions (Table III).

The protocol developed to solubilize the λ*cII*–β-globin fusion protein from *E. coli*[33] involves isolation and washing of inclusion bodies, solubilization in 8 *M* urea, and purification of the denatured protein before refolding and cleavage of the hybrid protein. Isolated inclusion bodies were solubilized in 8 *M* urea, 25 m*M* Tris-acetate, pH 5.0, 1 m*M* EDTA, and 1 m*M* DTT (urea buffer). The solubilized protein was applied to a CM-Sepharose column and the fusion protein eluted with a gradient of 0–0.2 *M* NaCl in urea buffer. The eluted protein was then subjected to gel

filtration on a Sephacryl S-200 column in 5 *M* guanidine-HCl, 5 m*M* Tris-HCl, pH 8.0, 1 m*M* EDTA, 1 m*M* DTT. The eluate from this column containing the fusion protein was dialyzed to remove denaturant and allow the hybrid protein to refold before cleavage with factor Xa as described in an earlier section.

In *E. coli* over 10 eukaryotic proteins have been expressed fused to λ*cII* via the factor Xa cleavage site.[34] In each case the process sequence was similar: solubilization (denaturation), purification in a denatured state, refolding, and cleavage. The level of purity required for efficient refolding differed and was found to be protein specific.

Interleukin 2 (IL-2) in *E. coli* is another example of an aggregated protein that has been purified in a solubilized, denatured form before refolding.[23] IL-2 contains three cysteine residues and the intramolecular disulfide bond between Cys-58 and Cys-105 in native IL-2 is essential for activity. Partial purification of the solubilized IL-2 facilitated analysis of reoxidation of the molecule during refolding and therefore allowed optimization of refolding conditions to give the maximum yield of correctly oxidized IL-2. Purification was effected by gel filtration in 6 *M* guanidine-HCl, 50 m*M* Tris-HCl, pH 8.5, 10 m*M* DTT using Superose-12 (FPLC) on an analytical scale and Sepharose CL-6B on a larger scale.

One final purification strategy to consider is that in which gene fusions are designed and constructed to facilitate purification. The principle of this strategy is to fuse the gene of interest to a sequence coding for a polypeptide which is selectively recognized by a matrix-bound ligand. A general approach developed by Sassenfeld and co-workers[32] was to produce C-terminal polyarginine fusions. Efficient purification of such fusions is possible using two-step cation-exchange chromatography. For example, urogastrone–polyarginine[32] expressed in *E. coli* is insoluble and can be solubilized by sonication of whole cells in 5 *M* urea, 40 m*M* Tris–acetate, NaOH, pH 9.5. The supernatant from centrifugation of such an extract, adjusted to pH 5.5, was subjected to chromatography on SP-Sephadex. The fusion protein and other cationic proteins bound and were eluted with a 0–300 m*M* NaCl gradient. After digestion of the polyarginine tail with carboxypeptidase B, urogastrone does not bind, while the other cationic proteins do. Therefore, the second cation-exchange step yields highly pure urogastrone. The buffer used for both cation-exchange steps was 5 *M* urea, 40 m*M* Tris–acetate, pH 5.5.

There are examples of this fusion protein purification strategy which predate the polyarginine fusion approach (e.g., β-galactosidase[35] and

[34] K. Nagai, H.-C. Thorgensen, and B. F. Luisi, *Biochem. Soc. Trans.* **16**, 108 (1988).
[35] M. Koenen, U. Ruther, and B. Muller-Hill, *EMBO J.* **1**, 509 (1982).

CAT[36]). However, the conformation of these polypeptide components of the hybrid are essential for selective recognition by the affinity ligand. Therefore, when CAT–hCT was found to be insoluble in *E. coli* and required 8 *M* urea to effect solubilization, affinity chromatography was not possible.[31]

There have been recent developments in the design of fusion proteins which facilitate purification utilizing maltose-binding protein[37] and glutathione *S*-transferase.[38] However, a central aim of each of these strategies is to obtain a fusion protein which is soluble in the cytoplasm or is secreted to the periplasm.

Concluding Remarks

The protocols described in this chapter illustrate the solubilization and refolding requirements of specific proteins. Purification and analysis of the proteins during and after these processes are of importance but are not considered here as they are discussed in detail in later chapters in this volume.

However, it is important to emphasize that certain features of the final protein products may be the direct results either of the expression mechanism or the methods used to recover active, soluble protein. These include

1. Authenticity of the N-terminus/C-terminus
2. Lack of posttranslational modification (e.g., glycosylation)
3. Modifications resulting from solubilization conditions (e.g., deamidation at high pH, Met oxidation, and conversion of cysteine to cysteic acid at low pH)
4. Conformational authenticity of the refolded molecule

These features will undoubtedly affect the activity of the final product, and their impact will depend on the application for which the proteins are required.

[36] A. D. Bennet, S. K. Rhind, P. A. Lowe, and C. C. G. Hentschel, U.K. Pat. No. GB 2140810 B (1983).
[37] C. di Guan, P. Li, P. D. Riggs, and H. Inouye, *Gene* **67**, 21 (1988).
[38] D. B. Smith and K. S. Johnson, *Gene* **67**, 31 (1988).